EFFECT OF SULFUR MUSTARD ON MAST CELLS
IN HAIRLESS GUINEA PIG SKIN

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ABSTRACT

The skin of 24 anesthetized hairless guinea pigs was exposed to saturated sulfur mustard (bis-2-chloroethyl sulfide; HD) for 5 and 7 minutes using 14-mm diameter vapor cups. Animals were euthanatized 24 hours after exposure and skin specimens taken for morphometric evaluation of granulated mast cells with an image analysis system (IAS). Tissue specimens were processed in paraffin, sectioned at $5\mu m$ and stained with Unna's stain for mast cells. The number of granulated mast cells and the area occupied by mast cell granules was determined. There were significantly fewer mast cells ($p < 0.05$) in either HD exposure group than in sham-exposed animals, with significantly fewer mast cells in the 7-minute than the 5-minute HD group. There were also significantly smaller areas occupied by granules in either HD exposure group than in sham-exposed animals. HD-induced lesions in the hairless guinea pig have shown signs of an inflammatory response, and with their granules of vasoactive histamine, mast cells might be expected to play a role in HD-induced injury. Changes in mast cells exposed to low sulfur mustard levels, as detected by an IAS, may serve as an early marker for cutaneous damage, which might not be as easily determined with routine light microscopy.

INTRODUCTION

The vesicating agent sulfur mustard (HD) has many effects on the skin, eyes and respiratory tract (1-11). The histopathology

41

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of cutaneous HD lesions indicates that vasoactive and chemotactic mediators are produced within exposed areas, starting a few hours after exposure (6). Skin from the hairless guinea pig has been used to evaluate the pathogenesis of the HD lesion and the efficacy of potential therapeutic compounds (1-5). HD-induced lesions in this animal model are accompanied by an inflammatory response, primarily seen as an influx of neutrophils (1). In both humans and other animal models, an infiltration of polymorphonuclear granulocytes and monocytes has been noted following cutaneous exposure to HD (6, 8-11). The major mediators to vascular changes preceding cellular infiltration in nonspecific inflammatory responses are histamine and kinins. Mast cells, with their granules of the vasoactive amines histamine and serotonin as well as the acid mucopolysaccharide heparin and small amounts of other peptides, might be expected to play a role in HD-induced injury in the hairless guinea pig model.

Image analysis systems have already been used to study cutaneous lesions (12-13). Pixels of both desirable and non-desirable features in a cutaneous image, when translated from color in a hematoxylin and eosin stained section to a digitized, gray level image by an image analysis system (IAS), may have the same gray level value (12). Thus, the use of a special histochemical stain, which selectively highlights the desired feature(s), and of selective light filtration to reduce background interference may enhance the IAS detection process.

This study was designed to determine if mast cell numbers or mast cell granulation is changed by exposure to sulfur mustard in hairless guinea pig skin, when evaluated by an IAS. Positive correlations between changes in mast cells and HD exposure could lead to routine evaluation as to the extent of cutaneous damage with the IAS, supplementing light histopathological findings. Changes in mast cells exposed to low sulfur mustard levels, as detected by the IAS, may also serve as an early marker for cutaneous damage, which might not be as easily determined with routine light microscopy.

MATERIALS AND METHODS

The skin of 24 anesthetized hairless guinea pigs was exposed to saturated sulfur mustard (bis-2-chloroethyl sulfide; HD) vapor for 5 and 7 minutes. These exposure times cause moderate and severe pathological changes, respectively (43.7% VS 91.6% incidence of microblisters) (1). Male [Crl:IAF/HA (hr/hr) BR Vaf/Plus®] euthymic hairless guinea pigs (Cavia porcellus), 260-560g, from the Newfield, NJ, breeding facility were used. Upon arrival, they were quarantined and screened for evidence of disease before use. They were maintained under an AAALAC

accredited animal care and use program. Guinea pigs were individually housed in plastic cages (Lab Products, Inc., Maywood, NJ) on shredded corn cob bedding (Bed O'Cobs, The Andersons, Industrial Products Division, Maumee, Ohio/Delphi, Indiana) which was changed three times per week. Guinea pigs were provided commercial certified guinea pig ration (Zeigler Bros., Inc., Gardners, PA) and tap water *ad libitum*. Animal holding rooms were maintained at $21°\pm2°C$ with $50\%\pm10\%$ relative humidity using at least 10 complete air changes per hour of 100% conditioned fresh air. All guinea pig cages were covered with filter paper (Remay #2016, Research Equipment Co., Inc., Bryan, TX) to minimize heat loss and maintained on a 12-hour light/dark full spectrum lighting cycle with no twilight.

As previously described, each animal had eight exposure sites with two sites each for 5- and 7-minute exposures (1). Hairless guinea pigs were anesthetized with an intramuscular combination of 30 mg/kg ketamine HCl (Vetalar, 100 mg/ml, Parke-Davis, Division of Warner-Lambert Co., Morris Plains, NJ) and 6 mg/kg xylazine (Rompun, 20 mg/ml, Mobay Corp., Animal Health Division, Shawnee, KS) and placed in sternal recumbency. Animals were draped to expose only the back area used in the experiment. The cranial and caudal boundaries of each exposure site were marked with an indelible pen to localize each site in the absence of visible changes. Two sites, one on either side of the dorsal midline, were set up for both 5- and 7-minute vapor exposures. Contralateral pairs of exposure sites were successively rotated on the animals to preclude anterior/posterior sensitivity biases. The HD vapor dose was established by varying the duration of skin exposure to the concentration of HD vapor generated under polyethylene caps 14 mm wide and 5 mm deep (No. P799C, Columbia Diagnostics, Inc, Springfield, VA). Caps were fitted with 14-mm discs of Whatman No. 2 filter paper, fixed 5 mm above the cap rim. The filter paper in each inverted cap was wet with 10 μl of neat HD. Previous studies (4) established this volume of HD as sufficient to wet the filter paper without run-off. Loaded caps were stored with the rims on glass microscope slides for at least 5 min prior to transfer onto animals to establish an HD vapor pressure. The vapor caps were held to the skin by double-sided tape assemblies. Although the HD vapor concentration under the caps was not determined it was estimated from the equilibrium vapor pressure (14) of 0.090 mm of Hg at 30°C. This corresponded to a vapor concentration of 770 mg/m^3 HD. After removal of caps and tape, the animals were placed in individual cages for observation. Animals remained anesthetized for a period of about 1.5 to 2 hr. After anesthesia the animals did not display any signs of distress or discomfort during the 24-hr observation period.

Twenty-four hours after exposure, euthanasia was performed using inhaled overdoses of Halothane (Halocarbon Laboratories,

Inc., Hackensack, NJ). Dermal skin punch (8 mm) specimens were taken from the center of each exposure site and fixed in 10% neutral buffered formalin. One 5-minute exposure site and one 7-minute exposure site per animal were evaluated for morphometric changes in mast cells, for a total of 24 sites examined per exposure group. A total of 32 exposure sites were taken and evaluated from 4 sham HD-exposed guinea pigs to serve as naive controls. (Vapor cups with no HD applied were held in place for 7 minutes.) All tissue specimens were embedded in paraffin, sectioned at $5\mu m$, and one section each stained with Unna's stain (15) for mast cells and hematoxylin and eosin (H&E/Gill's #3). The H&E stained sections were evaluated with routine light microscopy for pathological changes. The Unna's stained sections were evaluated morphometrically with a Quantimet 970 Image Analysis System (Leica Cambridge, Cambridge, England). An average area of $6.48 \times 10^5 \mu m^2$ of skin was examined per site. The first digitized image produced was processed through a $518.8\eta m$ interference filter; this allowed only the image of purple mast cell granules and a small amount of dark tissue processing debris to be detected. The first image was stored in the IAS computer's memory. A second digitized image, from the same field of view, was processed through a $440.0\eta m$ interference filter, allowing only the image of the dark tissue processing debris to be detected. The second image was subtracted from the first image, thus removing the tissue processing debris from the first image. Manual editing was used to remove concentrated stain droplets or processing debris not completely subtracted out as well as any keratohyalin granules in the epidermis or hair follicles picked up by the Unna's stain. The final edited image was used to count granulated mast cells and to determine (in μm^2) both the total area of detected granules and total area of skin evaluated. A granulated mast cell concentration was then calculated by dividing the number of mast cells detected by the total area evaluated (stated as a percentage). The number of pixels generated by the IAS represented the area in μm^2 occupied by mast cell granules. A "granularity index" was calculated by dividing the area of granules detected by the area examined, then multiplying by 100. The means of both the granulated mast cell concentrations and granularity indexes for all groups were tested for significant differences using an analysis of variance. Duncan's multiple range tests were then performed to test for significant differences among the 5- and 7-minute HD exposure groups and the sham-exposed animals. Comparisons were considered significant at the $p < 0.05$ level.

RESULTS

Granulated mast cells were frequently seen with routine light microscopy in the superficial papillary dermis of the skin sections not exposed to HD (Unna's stain). Mast cell granules

44

were prominently stained with the Unna's stain, with little other tissue staining. As seen by H&E, the sham-exposed group showed some epithelial cells undergoing dyskeratosis in one animal, and very few widely scattered inflammatory cells in the superficial dermis of two animals. These changes were considered insignificant, and these sections showed no evidence of HD injury. Histopathological lesions in HD-exposed skin sections (as seen by routine light microscopy using H&E), such as intracellular edema, basal cell necrosis, pustular epidermitis, microblister formation and follicular necrosis, are described in more detail elsewhere (1). The epidermis in the 5-minute HD exposure group had areas of intracellular edema and the dermis was infiltrated by neutrophils (H&E). In the 7-minute HD exposure group the epidermis had areas of necrosis, microvesicles and a marked inflammatory response (H&E). The few granulated mast cells that were present in the HD-exposed skin sections were predominately located in the deeper portions of the papillary dermis (Unna's stain).

Interference light filters used on the IAS further enhanced the contrast between Unna stained mast cell granules and background tissue, allowing the number of granulated mast cells to be counted by the IAS, as well as determination of the area fraction occupied by the granules. Significant differences in granulated mast cell concentrations and granularity indexes were found using Duncan's multiple range tests. There were significantly

FIGURE 1. Mean mast cell concentration per exposure. Significant differences ($p < 0.05$) were found between either the 5- or 7-minute HD exposure groups and the sham-exposed animals, as well as between the two HD-exposed groups.

fewer mast cells ($p < 0.05$) in either the 5- or 7-minute HD exposure groups than in the sham-exposed animals, with significantly fewer mast cells in the 7-minute HD than the 5-minute HD group. Mean mast cell concentrations are depicted in Figure 1. There were also significantly smaller areas occupied by granules in either the 5- or 7-minute HD exposure groups than in the sham exposure group. However, the difference in these indexes between the two HD exposure groups did not reach significance, possibly because some of the mast cells observed in
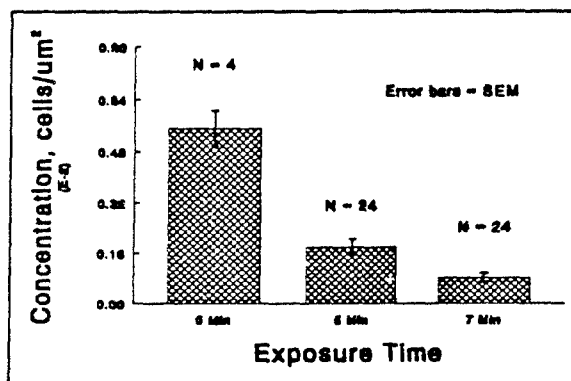
the 5-minute exposure group appeared to be partially degranulated. Mean granularity indexes are depicted in Figure 2.

DISCUSSION

Mast cells are reported to contribute to the acute inflammatory process (16-17). Thus, finding a way to quantitate mast cell participation in the HD-induced cutaneous inflammatory process may aid in understanding lesion development and serve as a marker for therapeutic intervention.

FIGURE 2. Mean granularity index per exposure. Significant differences ($p < 0.05$) were found between either the 5- or 7-minute HD exposure groups and the sham-exposed animals.

This study showed that granulated mast cell concentration decreased 24 hours after HD exposure in hairless guinea pig skin. The area occupied by mast cell granules was also decreased. The few remaining granulated mast cells were located primarily in the deeper portions of the papillary dermis, suggesting that mast cells closest to the epidermis have undergone the most degranulation. There appears to be a dose-related relationship between exposure time and mast cell concentration, as significant differences were noted among all three experimental groups.

Culture fluids from human explants, exposed to topically applied dilute HD contain increased amounts of histamine, plasminogen activator and usually prostaglandin E_2 (7). Mast cells were also counted and the amount of degranulation was subjectively graded in that study. Mast cell degranulation increased after the application of dilute HD, implying that increased histamine levels found in culture fluids was of mast cell origin (7). The current study indicates that mast cells play an important inflammatory role in the hairless guinea pig model.

In a screening program where large numbers of potential pretreatment and treatment compounds are to be evaluated, a mast cell evaluation procedure may be beneficial in addition to routine histopathological evaluation with light microscopy. Evaluation with an IAS may be a more sensitive way to quantitate
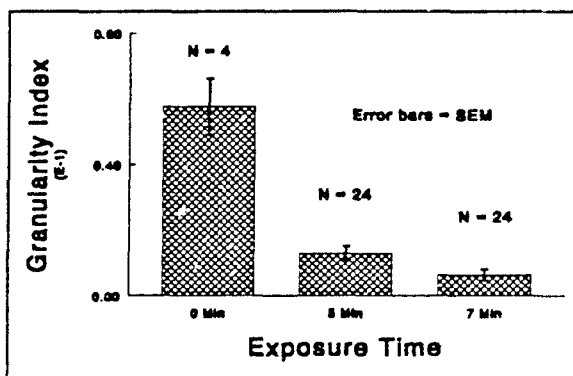
changes in mast cells participating in the inflammatory process. As such, evaluating mast cell changes where the HD exposure levels are low, or the changes due to potentially therapeutic compounds is slight, an IAS may aid greatly in determining if a change is significant. Additional studies evaluating longer and shorter exposure times, as well as varying post-exposure tissue collection times may further aid in understanding the relationship between HD-induced histopathological changes and mast cells in hairless guinea pig skin. Tne application of an IAS procedure to other cellular markers of the HD-induced inflammatory process, such as evaluation of neutrophils with a myeloperoxidase stain, may also prove useful.

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